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Synergistic effect of *Tim4* and *MFG-E8* null mutations on the development of autoimmunity

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Abbreviations used: MFG-E8, milk fat globule-EGF Factor 8; Tim, T-cell immunoglobulin- and mucin-domain-containing; SLE, systemic lupus erythematosus.

Abstract

Phagocytes, including macrophages, recognize phosphatidylserine exposed on apoptotic cells as an "eat me" signal. Milk Fat Globule EGF Factor VIII (MFG-E8) is secreted by one subset of macrophages, whereas Tim4, a type I-membrane protein, is expressed by another. These proteins bind tightly to phosphatidylserine on apoptotic cells and enhance their engulfment by macrophages. To study the contribution of these proteins to the engulfment of apoptotic cells, we established a mouse line that was deficient in the genes encoding MFG-E8 and Tim4. The null mutation of *Tim4* impaired the ability of resident peritoneal macrophages, but not thioglycollate-elicited macrophages, to engulf apoptotic cells. Mice deficient in either *MFG-E8* or *Tim4* on the C57BL/6 background developed hardly any autoantibodies, but aged female mice deficient in both *MFG-E8* and *Tim4* developed autoantibodies in an age-dependent manner. TNFα is known to protect against systemic lupus erythematosus-type autoimmunity, while type I IFN accelerates the disease. Indeed, the administration of an anti-TNFα antibody or a reagent that stimulates the IFNα production [2,6,10,14-tetramethylpentadecane (TMPD; also known as pristane)] enhanced the production of autoantibodies in the *MFG-E8*- and *Tim4*-double-deficient mice. These results suggest that the double deficiency of Tim4 and MFG-E8, phosphatidylserine-binding proteins, can trigger autoimmunity and that TNFα and type I IFN regulate reciprocally the development of autoimmune disease.

Introduction

Everyday, 10^8 cells in the human body undergo apoptotic cell death (1). At the same time, several billion red blood cells are produced, and an equal number of nuclei (pyrenocytes) is expelled from erythroblasts in this process (2). The dead cells and expelled nuclei must be swiftly removed to prevent them from releasing noxious materials. Failure to remove them appears to activate the immune system and lead to systemic lupus erythematosus (SLE)-type autoimmune diseases (3,4).

Macrophages, which are professional phagocytes, are responsible for clearing apoptotic cells and pyrenocytes (1). Macrophages recognize phosphatidylserine exposed on the plasma membranes of the dead cells and pyrenocytes as an "eat me" signal, and engulf them for degradation in the lysosomes (5,6). We and others previously identified several molecules that bind phosphatidylserine prior to resulting in the engulfment of apoptotic cells (6-11). Among them, MFG-E8 (Milk Fat Globule EGF Factor VIII) is a soluble protein secreted from a subset of macrophages and immature dendritic cells, including thioglycollate-elicited peritoneal macrophages, GM-CSF-induced bone marrow-derived dendritic cells, and Langerhans cells in the skin (12). MFG-E8 acts as bridge between dead cells and phagocytes, binding to phosphatidylserine on apoptotic cells and to integrins $\alpha_v\beta_3$ or $\alpha_v\beta_5$ on macrophages or immature dendritic cells (10).

Tim (T-cell immunoglobulin- and mucin-domain-containing)-4 is a type I membrane protein that is expressed by other subsets of macrophages, including resident peritoneal

macrophages and splenic MOMA-1^{+}-marginal zone macrophages (11,13). Tim4 binds tightly to phosphatidylserine *in vitro* and supports the engulfment of apoptotic cells when expressed in mouse NIH3T3 cells.

We previously showed that *MFG-E8*-deficient mice on a 129/B6-mixed background produce autoantibodies and suffer from autoimmune diseases (14). On the other hand, two groups recently showed that *Tim4*-deficient mice on the 129 or B6-background developed little or no autoimmunity (13,15). To clarify the role of MFG-E8 and Tim4, here we established a mouse line deficient in the genes for both *MFG-E8* and *Tim4* on the B6-background. Mice deficient in either *MFG-E8* or *Tim4* did not develop autoantibodies. In contrast, the *MFG-E8*/*Tim4*-double deficient mice developed a high level of autoantibodies, and this process was accelerated by the administration of an anti-TNFα neutralizing antibody or the hydrocarbon oil 2,6,10,14-tetramethylpentadecane (TMPD; also known as pristane) that stimulates type I IFN production. These results suggested that mice deficient in both *MFG-E8* and *Tim4,* expressed in different sets of the macrophages, may generate sufficient numbers of unengulfed apoptotic cells to activate the autoimmunity, and TNFα and type I IFN reciprocally regulate the process.

Methods

Mice

C57BL/6 mice were from Japan SLC (Shizuoka, Japan). *CAD*$^{-/-}$ mice were described (16). *MFG-E8*$^{-/-}$ mice generated from a 129 ES cell line (14), were backcrossed to the C57BL/6 mice eight times. *Tim4*$^{-/-}$ *MFG-E8*$^{-/-}$ mice were generated by crossing *Tim4*$^{+/-}$ *MFG-E8*$^{-/-}$ mice. All mice were housed in a specific pathogen-free facility at Kyoto University Graduate School of Medicine. All animal experiments were carried out in accordance with protocols approved by the Kyoto University Animal Care and Use Committee.

Monoclonal antibodies and reagents

To produce an mAb against Tim4, Armenian hamsters were immunized with Tim4-Fc, in which the extracellular region of mouse Tim4 was joined to human IgG Fc region (11). Lymphocytes from the immunized hamsters were fused with a mouse myeloma NSO^{bcl2} (17). One hybridoma (Mat4-9-5) that was suitable for immunohistochemical staining, FACS analysis, immunoprecipitation, Western blotting, and neutralization of the engulfment was grown in serum-free GIT medium (Nihon Pharmaceutical, Tokyo, Japan), and the secreted mAb was purified using Protein A-Sepharose. The hamster Kat5-18 mAb was described (11). A rat anti-TNFα mAb (clone: MP6-XT22) (18) was produced by culturing the hybridoma in GIT medium, and purified by fractionation with $(NH_4)_2SO_3$-precipitation. A leucine zipper-containing human Fas ligand (FasL) was

prepared in 293T cells, and partially purified as described (19).

Targeted disruption of the Tim4 gene

The *Tim4*-targeted mice were generated as custom order by Unitech (Kashiwa, Chiba, Japan). In brief, the *Tim4* chromosomal gene was isolated from BAC clones of C57BL/6 mice, and its exon 1 and most of exon 2 were replaced by a coding sequence for Venus and *neo* gene (Fig. 1). To increase the efficiency of homologous recombination, a coding sequence for the diphtheria toxin A fragment (DT-A) was inserted downstream of exon 5 in the targeting vector. The targeting vector was transfected into ES cells (clone: Bruce 4) derived from C57BL/6 mice (20). The ES clones carrying the *Tim4*-deficient allele were introduced into host embryos, and chimeric mice were produced. The chimeric mice with a high ES cell contribution were crossed with C57BL/6 mice to obtain heterozygous *Tim4*-deficient mice.

The genotype of the *Tim4* and *MFG-E8* genes was determined by PCR using TaKaRa LA Taq (Takara Bio, Kyoto, Japan) or Taq DNA polymerase from Ampliqon (Skovlunde, Denmark). For the wild-type and mutant alleles of the *Tim4* gene, a sense primer specific for the wild-type (5'-ATTGAGGGAGGTCATTCAGGA-3') or mutant allele (5'-GACCCTGAAGCTGATCTGCA-3') was used with a common antisense primer (5'-AGCGCACATTCTTCTTGACA-3'). The wild-type and mutant alleles of the *MFG-E8* gene were detected by a similar method using the wild-type

(5’-CTTTGGAGGATGTACACAGA-3’) or mutant (5’-CGTGGGATCATTGTTTTTCT-3’) specific sense primer and the common antisense primer (5’-CTTTATGTACTGTGCCTCCA-3’).

Administration of pristane and anti-TNFα monoclonal antibody.

Female mice at 7-10 weeks of age were injected *i.p.* with 0.5 ml Pristane (Sigma-Aldrich, , St. Louis, MO) as described (21), while the anti-TNFα mAb or control rat IgG (200 μg/head) was administered twice a week by *i.p*. injection as described (22).

Isolation of tissue macrophages

Resident and thioglycollate-elicited peritoneal macrophages were isolated from female C57BL/6 mice at 6 weeks of age as described (10,11). Alveolar macrophages were prepared from the lungs by bronchoalveolar lavage with PBS containing 0.5 mM EDTA, cultured on plastic dish for 2 h, and washed with PBS to remove non-adherent cells. Kupffer cells were prepared as described (23). In brief, the liver was perfused *in situ* at a flow rate of 5 ml/min for 5 min with prewarmed (37°C) Hank’s Balanced Salt Solution (HBSS, Invitrogen, Carlsbad, CA) containing 0.5 mM EGTA, then with HBSS containing 1.8 mM $CaCl_2$, 2.0 mM $MgCl_2$, 1.0 mg/ml collagenase D, and 0.1 mg/ml DNase I for 5 min. The liver was removed and minced at 4°C with scissors in 10 ml HBSS. After filtrating through stainless steel mesh (diameter: 106 μm), the cell suspension was centrifuged at 50

x *g* for 1 min at 4°C to remove aggregates. The cells in the supernatant were collected by centrifugation at 280 x *g* for 10 min, and suspended in cold HBSS containing 10% FCS. The cells were then layered on 17% metrizamide (Sigma-Aldrich), and centrifuged at 1800 x *g* for 30 min at 4°C. The cells at the interphase was collected, suspended in HBSS containing 10% FCS, and precipitated by centrifugation at 780 x *g* for 10 min. Cells were suspended in serum-free RPMI, cultured at 37°C for 15 min in non-coating dish (Iwaki, Chiba, Japan), and washed to remove non-adherent cells.

Flow cytometry

Cells were incubated on ice for 30 min with 1 μg/ml biotinylated Kat5-18 in 100 μl staining solution (PBS containing 2% FCS), followed by incubation with 2 μg/ml APC-Cy7-conjugated streptavidin (BD Bioscience, Franklin Lakes, NJ) and 2 μg/ml APC-conjugated anti-CD11b (BioLegend, San Diego, CA). The cells were then stained with 0.5 μM SYTOX Blue (Invitrogen) to exclude dead cells, and analyzed by flow cytometry with a FACSAria (BD Bioscience).

Phagocytosis assay

The engulfment of apoptotic cells was assayed by TUNEL staining with *CAD*$^{-/-}$ thymocytes as prey as described (10). In brief, 1 x 10^6 peritoneal cells were incubated in 12-well plates at 37°C for 2 h, and washed with PBS. Thymocytes from *CAD*$^{-/-}$ mice were induced to

undergo apoptosis by treatment with 4 units/ml FasL for 2 h, and 5 x 10^6 apoptotic cells were cultured at 37°C for 30 min with the peritoneal cells. After staining with APC-anti-CD11b, the cells were fixed with 1% paraformaldehyde, subjected to TUNEL staining with FITC-labeled dUTP (Roche Diagnostics, Indianapolis, IN), and analyzed by flow cytometry on a FACSAria (BD Biosciences). In some cases, 2 x 10^5 peritoneal cells were cultured in glass dishes (Iwaki) that had been coated with 1.0% gelatin. Mouse thymocytes were labeled with CellTracker™ Orange (Invitrogen), treated with FasL as described above, and apoptotic cells (1 x 10^6) were co-incubated with resident peritoneal macrophages for 30 min. After staining with FITC-anti-CD11b, the cells were observed by fluorescence microscopy (BioRevo BZ-9000, Keyence, Osaka, Japan).

Real-time PCR

Total RNA was prepared using ISOGEN (NIPPON GENE, Tokyo, Japan), followed by RNeasy Micro Kit (QIAGEN, Hilden, Germany), and was reverse-transcribed using Superscript III (Invitrogen). Aliquots of the products were amplified in a reaction mixture containing LightCycler™ 480 SYBR Green I Master (Roche Diagnostics). The primers used for real-time PCR were as follows. Tim4, 5'-GGCTCCTTCTCACAAGAAACCACA and 5'-TCAGCTGTGAAGTGGATGGGAGA; MFG-E8, 5'-GATCTTTCCAACAACCTAGCCTCC and 5'-ACCGCTTTCATCCTGGATGAACTC; GAPDH, 5'-AACGACCCCTTCATTGAC and 5'-TCCACGACATACTCAGCAC.

Solid-phase ELISA

The serum level of anti-dsDNA antibodies and anti-nuclear antibodies (ANA) was determined as described (24). In brief, for the anti-dsDNA, the linearized plasmid DNA (5 μg/ml) was immobilized on NucleoLink plates (Nalge Nunc, Rochester, NY) by treating it at 50°C for 5 h with 10 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in 10 mM 1-methylimidazole. The plates were washed with 5 x SSC containing 0.25% SDS, and nonspecific binding sites were blocked with 1% BSA in PBS. The serum was diluted to the appropriate concentration, added to the wells, and incubated at room temperature for 1 h. After washing with PBS containing 0.1% Tween 20, the concentration of autoantibodies bound to each well was quantified by ELISA with AP-conjugated rabbit anti-mouse Ig (Dako, Copenhagen, Denmark). Phosphatase activity was detected using the BluePhos Microwell Phosphatase Substrate System (KPL, Gaithersburg, MA) and quantified by measuring the absorbance at 620 nm. The ANA level was determined using the Mesacup EIA system from MBL (Nagoya, Japan) for human ANA, except that the HRP-conjugated goat anti-human Ig was replaced by AP-conjugated rabbit anti-mouse Ig and the plate was incubated with the mouse serum for 2 h.

Results

Requirement of Tim4 by peritoneal macrophages for the engulfment of apoptotic cells

In addition to Tim4 and MFG-E8, several molecules, such as BAI1, stabillin-2, Tim1, and Tim3 are reported to recognize phosphatidylserine and mediate the engulfment of apoptotic cells (8,9,11,25). We previously showed that MFG-E8 and Tim4 are rather complementarily expressed in different sets of macrophages. That is, Tim4 is expressed in resident peritoneal macrophages, while MFG-E8 is expressed in thioglycollate-elicited peritoneal macrophages (11). This was further confirmed with other tissue macrophages. Alveolar macrophages expressed MFG-E8 but not Tim4, while Kupffer cells expressed Tim4 but not MFG-E8 (Supplementary Fig. 1).

We previously established *MFG-E8*-null mice, and showed that MFG-E8 is required for the efficient engulfment of apoptotic cells *in vitro* by thioglycollate-elicited macrophages, and *in vivo* by the tingible-body macrophages in the spleen (14). To examine the role of Tim4 in the engulfment of apoptotic cells by a distinct set of macrophages, we established a *Tim4*-deficient mouse line (Fig. 1A). As shown in Figure 1B, about 40-50% of the cells in the mouse peritoneum was strongly stained with CD11b, and most of them expressed Tim4. As expected, the peritoneal cells from *Tim4*-null mice did not stain with anti-Tim4 mAb. When resident peritoneal macrophages were co-incubated with apoptotic thymocytes, they efficiently engulfed them (Fig. 2). The *Tim4*-null mutation almost completely abolished this ability. In agreement with MFG-E8 not being expressed in

resident peritoneal macrophages (11), the null-mutation of the *MFG-E8* gene did not have any effect on these cells' ability to engulf apoptotic cells. These results indicated that Tim4 plays an indispensable role in the engulfment of apoptotic cells in resident peritoneal macrophages.

Synergistic effect of MFG-E8 and Tim4 on the development of autoimmunity.

We previously established *MFG-E8*-null mice in a 129/B6 mixed background and showed that they, in particular the females, age-dependently developed an SLE-type autoimmunity (14). On the other hand, when mice bearing this mutation were back-crossed to C57BL/6 mice more than 8 times, this phenotype and the level of autoantibodies in the serum were significantly reduced (Fig. 3). Recently, two groups established *Tim4*-null mice in the 129 and B6 mouse strains, respectively. The *Tim4*-null mice on the 129 background do not produce autoantibodies (13), but those on the B6 background produce a low but significant level of them (15).

As shown in Figure 3, the *Tim4*-null mice that we established on the B6 background did not produce a significant level of autoantibodies (anti-dsDNA or ANA). We previously reported that the injection into B6 mice of recombinant MFG-E8 mutant (D89E), which masks the phosphatidylserine on apoptotic cells, causes strong autoimmunity (26). As discussed above, Tim4 and MFG-E8 are expressed in distinct sets of macrophages and probably function non-redundantly. We therefore examined whether mice bearing double

knockout mutations of the *MFG-E8* and *Tim4* genes would exhibit any effect on the development of autoimmunity. As shown in Figure 3, no autoantibodies (anti-ds DNA or ANA) were found in the serum of the *MFG-E8*/*Tim4*-double deficient mice at 10 weeks of age. On the other hand, by 30 weeks of age, the female *MFG-E8*/*Tim4*-double deficient mice developed a significant level of autoantibodies. These results suggested that the dose of unengulfed apoptotic cells generated by the lack of either the *Tim4* or *MFG-E8* gene alone, but not by their double deficiency, might be insufficient to trigger the development of autoimmunity.

Reciprocal regulation of the autoimmunity by TNFα and type I IFN

Previous studies by a number of groups showed that an SLE-type autoimmunity is reciprocally regulated by TNFα and type I IFN in mouse and human (27-31). That is, blocking the circulating TNFα or increasing IFNα level in the serum accelerates the development of the disease. To examine whether or not TNFα has any effects on the regulation of the SLE-type disease in the *Tim4*$^{-/-}$, *MFG-E8*$^{-/-}$ and *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ mice, a TNFα-neutralizing mAb was administered twice a week to these mice. As shown in Figure 4, a significant level of anti-dsDNA antibodies could be detected even in the serum of Tim4 or MFG-E8-single null mice after 6 weeks, and an enhanced synergistic effect on the development of the autoimmunity was observed in the double *MFG-E8*/*Tim4*-deficient mice. While, rat control IgG had no effect on autoimmunity in *Tim4*$^{-/-}$ mice (data not

shown). These results confirmed that the constitutive exposure to TNFα prevented the development of autoimmunity in these mice.

When pristane is introduced into mouse peritoneal cavity, it stimulates the production of type I IFN in a TLR-dependent manner, causing chronic inflammation (32). To examine the effect of type I IFN on the development of the autoimmune disease in *Tim4*$^{-/-}$, *MFG-E8*$^{-/-}$ and *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ mice, pristane was injected *i.p* into these mice at 7-10 weeks of age. As shown in Figure 4B, a high level of anti-dsDNA could be detected at 7 week after the pristane-injection in the *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ double-deficient mice. These data suggested that the pristane-induced inflammation, probably type I IFN system, accelerated the production of the autoantibody in *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ mice.

Discussion

A failure of the efficient engulfment of apoptotic cells has been believed to cause autoimmune disease, because noxious materials are released from unengulfed dead cells that undergo secondary necrosis (1,33). Many molecules have been proposed to regulate the engulfment of apoptotic cells, and mice with a deficiency of MER (a tyrosine kinase receptor) developed a severe autoimmune response, including splenomegaly, proteinuria, and elevated autoantibodies (34). Here, we showed that, although a single deficiency of the *MFG-E8* or *Tim4* gene did not activate autoimmunity in the B6 mouse strain, the double-deficiency of the *Tim4* and *MFG-E8* genes did. This finding is different from our previous one showing that *MFG-E8*-null mice develop autoimmunity (14). We now attribute this observation to the mixed 129/B6 background of the *MFG-E8*-null mice that we analyzed in the earlier study, because the mouse strain of 129 and B6 chimeric background often spontaneously develops autoimmunity (35). The experiments in this report were therefore performed with *MFG-E8*-null mice that were backcrossed at least 8 times to B6 mice. Our results also do not agree with Rodriguez-Manzanet *et al* (15), which showed a significant development of autoantibodies in *Tim4*-null mice on the B6 background. Because SLE-type autoimmunity is greatly influenced not only by genetics but also by environmental factors (36), it is possible that, although our mice and those studied by Rodriguez-Manzanet *et al* (15) were kept under specific pathogen-free conditions, some small difference in the environment might result in a different outcome in the development

of the autoimmunity.

In any case, we showed here that the double mutation of the *MFG-E8* and *Tim4* genes activated autoimmunity with high frequency. MFG-E8 and Tim4 are expressed in different sets of macrophages, and are required for the engulfment of apoptotic cells differentially among macrophages. Tim4 was reported to stimulate proliferation of T cells (37). However, no apparent abnormality (splenomegaly, lymphadenopathy, or lymphopenia) was observed in the Tim4-null mutation. We also did not observe unengulfed apoptotic cells in *Tim4*-, *MFG-E8*-, and *MFG-E8/Tim4*-double deficient mice. Whereas, our preliminary analysis of the dexamethasone-treated mice suggested that the *MFG-E8/Tim4*-double deficient mice may have the reduced ability to clear apoptotic thymocytes (K.S. and S.N., unpublished observations). Thus, although a possibility that the abnormal T cell development caused by the Tim4-deficiency enhances the autoimmunity cannot be ruled out, we postulate that the double mutations of the *Tim4* and *MFG-E8* genes may generate a higher dose of unengulfed apoptotic cells that can be immunogenic. This appears to agree with the hypothesis that autoimmunity develops only when the determining "factors" for SLE reach a certain threshold (38).

Resident peritoneal macrophages express Tim4, and require Tim4 to engulf apoptotic cells. MER is also expressed in mouse resident peritoneal macrophages, and seems to be indispensable for this ability (39), suggesting that Tim4 and MER co-operate in the engulfment of apoptotic cells. On the other hand, tingible body macrophages in the spleen

express Tim4, MFG-E8, and MER, although MFG-E8 and MER, but not Tim4, seem to be required for the engulfment of apoptotic cells by tingible body macrophages (40). However, it is not clear how MFG-E8, Tim4 and MER in different macrophages trigger the engulfment of apoptotic cells. To fully understand the molecular mechanism for the recognition of apoptotic cells, it will be necessary to reconstitute the engulfment system with these and other phosphatidylserine-binding proteins.

TNFα is a pleiotropic cytokine with many physiological and pathological functions (41). It is responsible for the inflammation caused by endotoxin-induced septic shock and rheumatoid arthritis. On the other hand, the chronic exposure of mice to a low level of TNFα suppresses immune reactions, for example by inhibiting cytokine production from T cells and activating regulatory T cells (42). The administration of an anti-TNFα mAb to human patients with rheumatoid arthritis sometimes triggers an SLE-type autoimmune disease (43). Similarly, the administration of the anti-TNFα mAb accelerated the development of autoantibodies in *MFG-E8*-, *Tim4*-, and *MFG-E8*/*Tim4*-double deficient mice, supporting the previous report that TNFα inhibits the development of SLE (44). *Tim4*$^{-/-}$ macrophages were reported to produce TNFα constitutively (13). Although we could not detect the difference in the serum TNFα level between the wild-type and *Tim4*$^{-/-}$ mice (data not shown), it is possible that the local chronic exposure of the *Tim4*$^{-/-}$ immune system to a low concentration of TNFα may explain the difference between the lack of autoantibody production in *Tim4*$^{-/-}$ mice and the strong autoantibody production in adult

mice treated with an anti-Tim4 mAb (11). The 3'-non coding region of *TNFα*, which has a regulatory role in its gene expression, shows a strong polymorphism among mouse strains, which may explain the different susceptibilities of various *MFG-E8*-null mouse strains to autoantibody production.

IFNα, a type I IFN, has recently been recognized as a critical mediator of human SLE (30,31). Administration of pristane into mice induces the IFN gene expression leading to the development of SLE-type autoimmune disease in a Toll-like receptor (TLR)7-dependent manner (21). TLR7 is a receptor for nucleic acids. Since the mutations (*lpr* and *gld*) in Fas and Fas ligand that are involved in the activation-induced cell death of lymphocytes (45) reduces the pristane-induced SLE phenotype (46), it is believed that the nucleic acids released from dead cells trigger the disease (32). The accelerated development of the pristane-induced autoimmunity in *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ mice may support the idea that the lack of Tim4 and MFG-E8 generates more unengulfed dead cells, which undergo secondary necrosis to release nucleic acids. Ninety percent of human SLE patients are female (47), and an involvement of female hormone is considered to play a role. Like human patients, the SLE phenotype in *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ mice is prevalent in female, indicating that the *Tim4/MFG-E8*-null mice developed for this report may be useful for studying SLE-type autoimmune diseases.

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Legends to Figures

Fig. 1. Establishment of *Tim4*-deficient mice. (A) Targeting of the *Tim4* gene. Schematic representation of the wild-type and mutant loci of the *Tim4* gene and the targeting vector. Exons are represented by closed boxes. The targeting vector carries the neomycin resistance gene (neo) and the gene encoding the Diphtheria toxin A fragment (DT-A). (B) Expression of Tim4 in Mac-1^+ peritoneal cells. Peritoneal cells from the wild-type and *Tim4*-deficient mice were stained with biotin-labeled anti-Tim4 followed by APC-Cy7-streptoavidin and APC-CD11b, and analyzed on a FACSAria.

Fig. 2. Phagocytosis of apoptotic cells by resident peritoneal macrophages. (A) Resident peritoneal macrophages from wild-type, *Tim4*$^{-/-}$, *MFG-E8*$^{-/-}$ and *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ mice were incubated at 37°C for 30 min with apoptotic *CAD*$^{-/-}$ thymocytes, and the phagocytosis was assayed by TUNEL staining followed by FACS analysis. The percentage of TUNEL-positive cells in the Mac-1^+ population is plotted. (B) Resident peritoneal macrophages from wild-type, *Tim4*$^{-/-}$, *MFG-E8*$^{-/-}$ and *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ mice were incubated at 37°C for 30 min with Celltracker^{TM} Orange-labeled-apoptotic thymocytes, washed with PBS, stained with FITC-anti-CD11b, and observed by fluorescence microscopy.

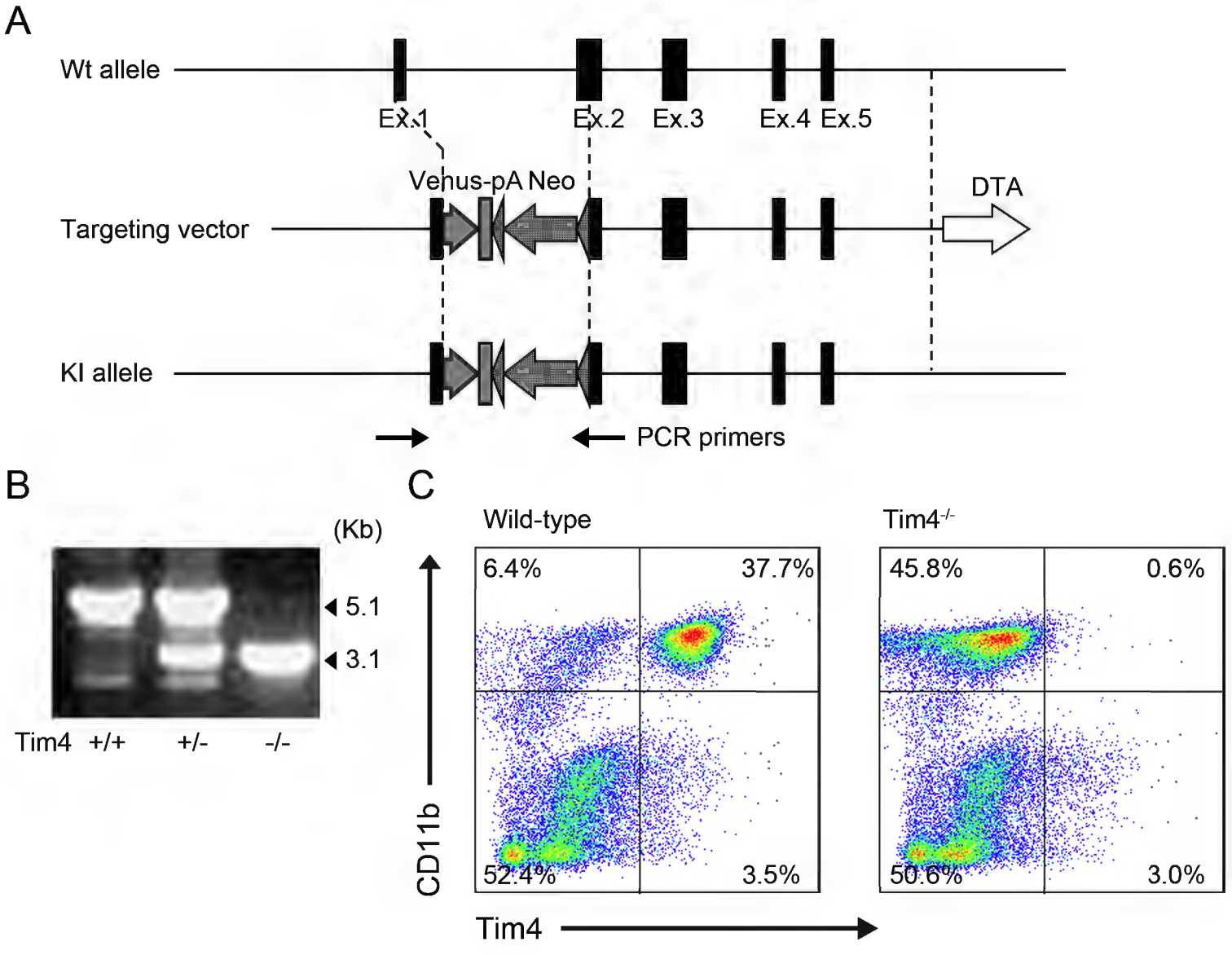
Fig. 3. Production of autoantibodies in *MFG-E8*$^{-/-}$*Tim4*$^{-/-}$ mice. The concentration of the

anti-dsDNA antibody (A and B) and ANA (C and D) was determined for the male and female mice of the wild-type, *Tim4*$^{-/-}$, *MFG-E8*$^{-/-}$, and *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ genotypes at 10 (A and C) and 30 (B and D) weeks of age. The average value was determined for each group (more than 5 mice per group), and is shown as bar. The *p*-values between the indicated pairs are shown.

Fig. 4. Enhanced production of the autoantibodies. (A) Effect of anti-TNFα mAb on the autoantibody production. Wild-type, *Tim4*$^{-/-}$, *MFG-E8*$^{-/-}$, and *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ female mice (5 mice each) at 7-11 weeks of age were injected *i.p.* twice a week with the anti-TNFα mAb (200 μg per head). Before the injection (0 weeks) and at 2, 4, and 6 weeks after the injection, the anti-dsDNA antibody level of the serum was determined. The average values are indicated by horizontal bars, and the *p*-values for the differences between the indicated pairs are shown. (B) Effect of pristane on the autoantibody production. Wild-type, *Tim4*$^{-/-}$, *MFG-E8*$^{-/-}$, and *Tim4*$^{-/-}$*MFG-E8*$^{-/-}$ female mice (5-7 mice each) at 7-10 weeks of age were injected *i.p.* with pristane (0.5 ml per head). Before and at 7 weeks after the injection, the serum was collected, and the level of the anti-dsDNA antibody was determined. Bars indicate the mean values.

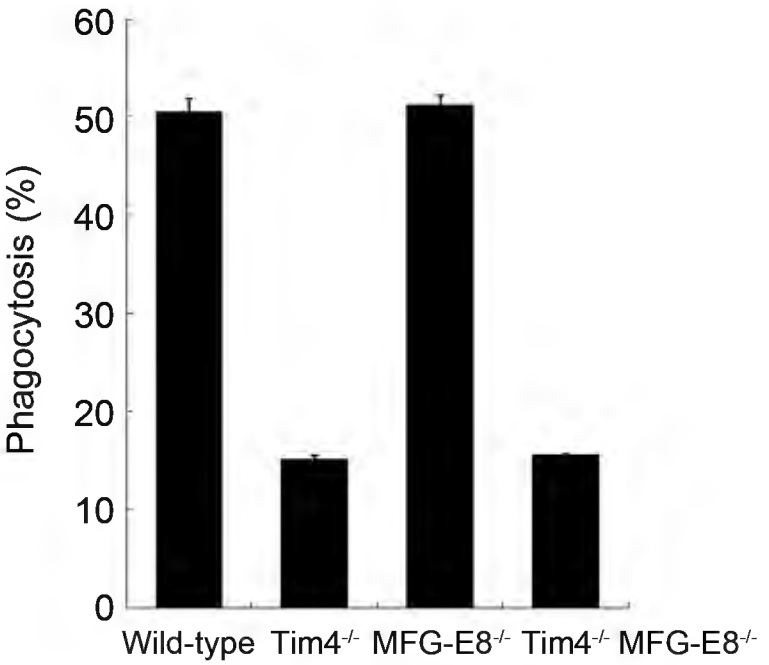
Supplemental Fig. 1. Expression of Tim4 and MFG-E8 in different macrophages. RNA was prepared from the resident peritoneal macrophage, thioglycollate-elicited peritoneal

macrophage, alveolar macrophage, and Kupffer cells. The mRNA level for MFG-E8 and Tim4 was analyzed by real-time PCR, and is expressed as relative to the GAPDH mRNA level.



Miyanishi et al. Figure 1

A



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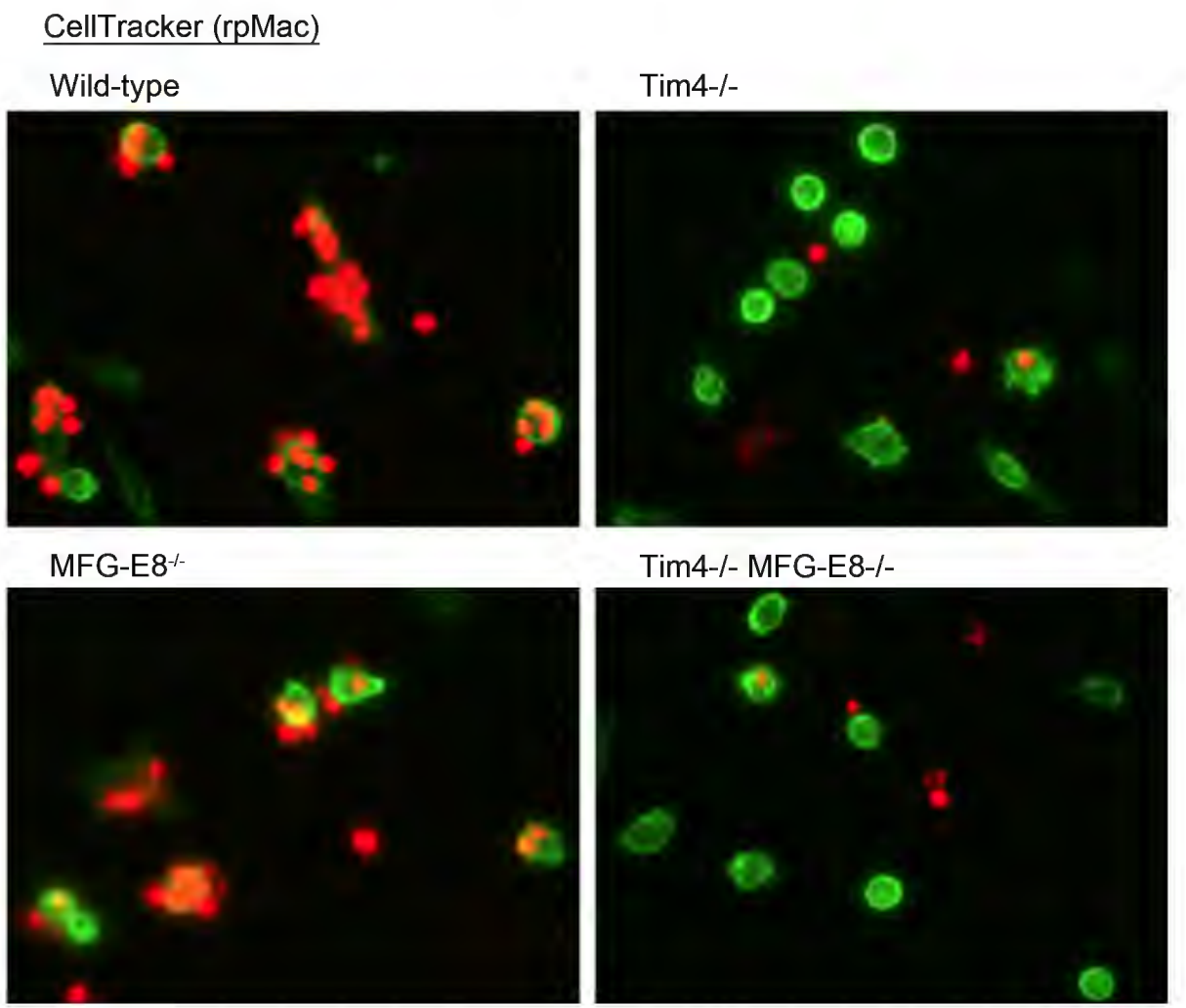
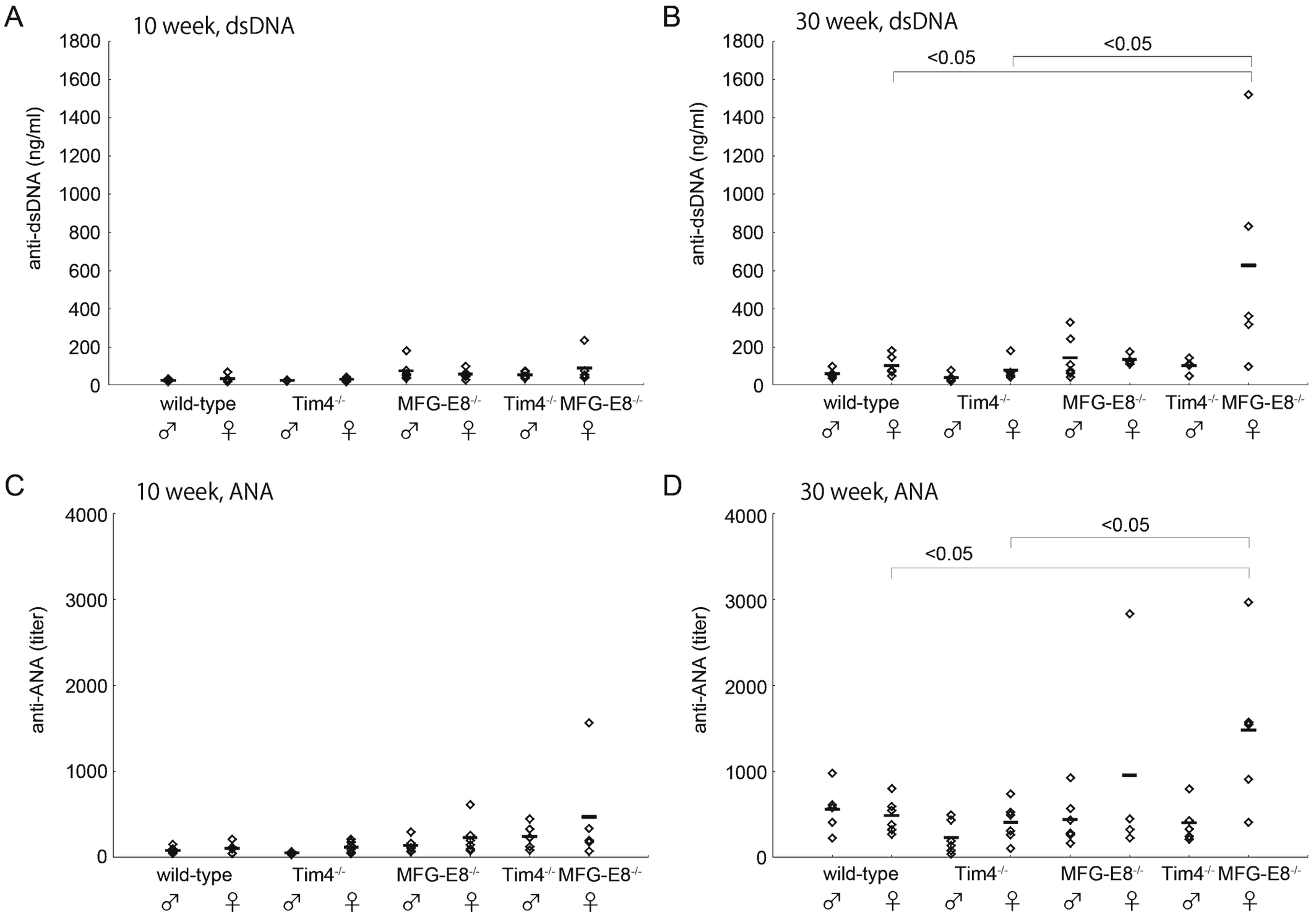
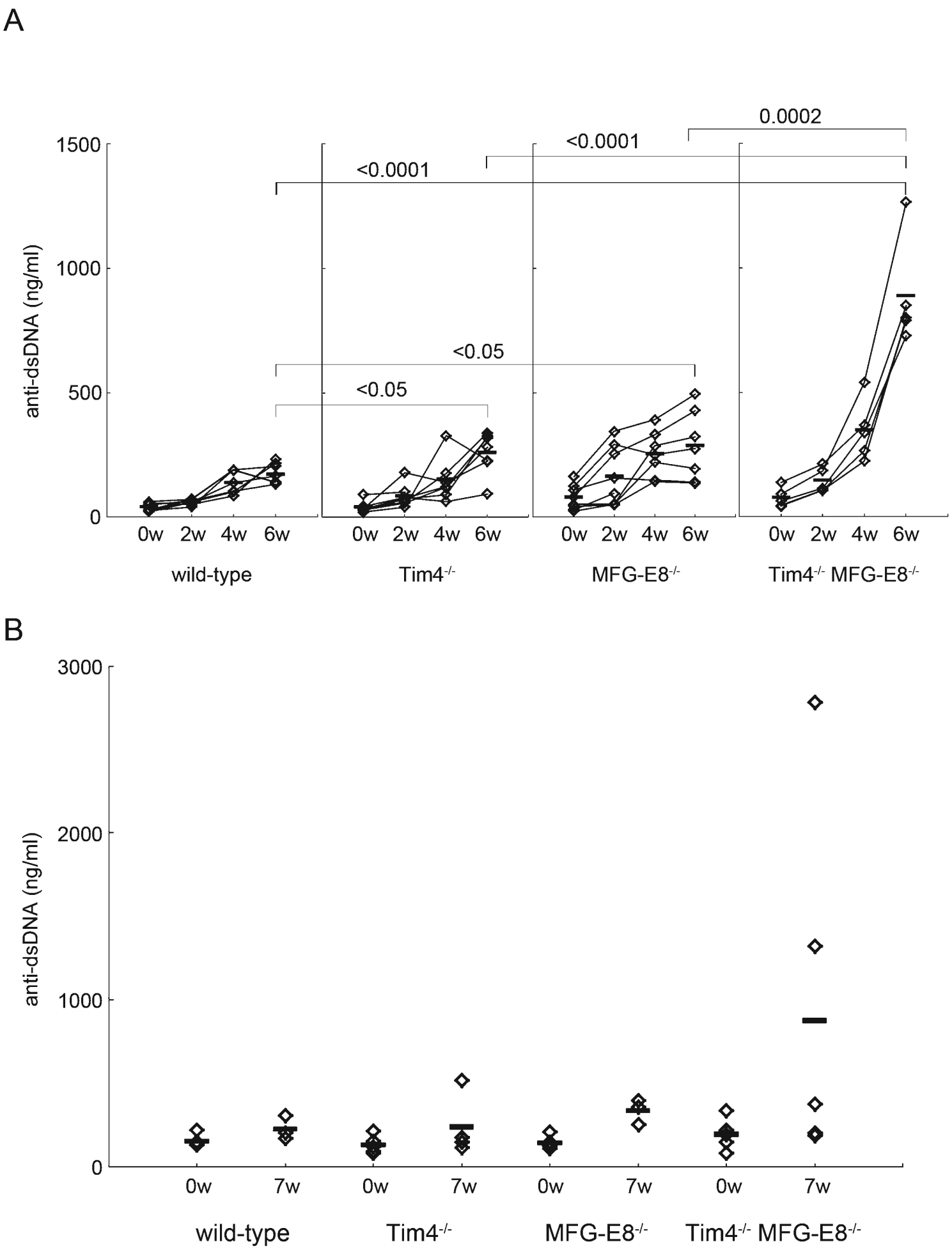


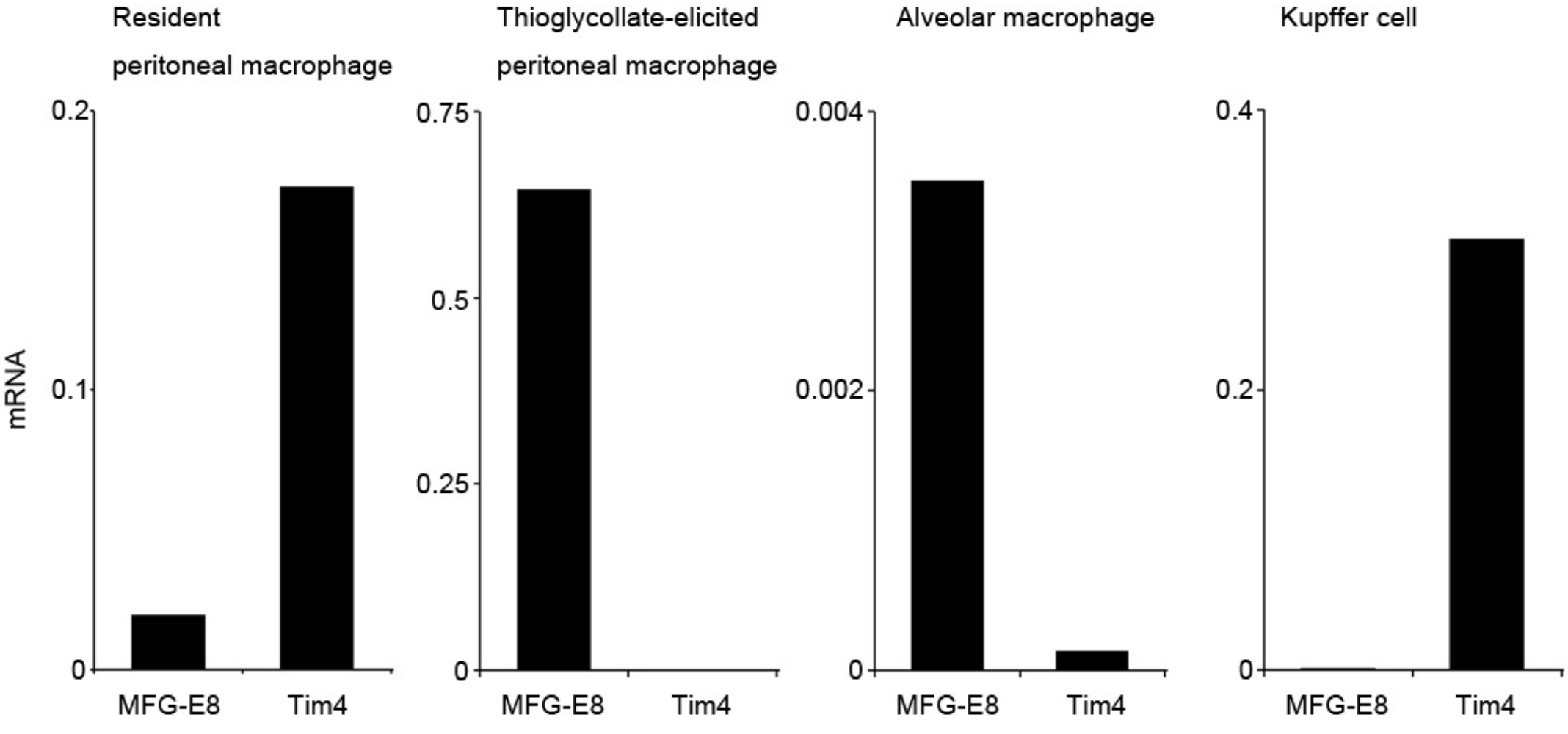
Miyanishi et al. Figure 2



Miyanishi et al. Figure 3



Miyanishi et al. Figure 4



Miyanishi et al. Supplementary Figure 1